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Sucrose Substitutes Affect the Cariogenic Potential of *Streptococcus mutans* Biofilms

S.C. Durso[a] L.M. Vieira[a] J.N.S. Cruz[a] C.S. Azevedo[b] P.H. Rodrigues[a]
M.R.L. Simionato[a]

[a]Department of Microbiology, Institute of Biomedical Sciences, and [b]Department of Operative Dentistry, Dental School, University of São Paulo, São Paulo, Brazil

Key Words
Biofilm · Demineralization · Optical coherence tomography · Reverse transcription quantitative polymerase chain reaction · Sorbitol · Sucralose · *Streptococcus mutans*

Abstract
Streptococcus mutans is considered the primary etiologic agent of dental caries and contributes significantly to the virulence of dental plaque, especially in the presence of sucrose. To avoid the role of sucrose on the virulence factors of *S. mutans*, sugar substitutes are commonly consumed because they lead to lower or no production of acids and interfere with biofilm formation. This study aimed to investigate the contribution of sugar substitutes in the cariogenic potential of *S. mutans* biofilms. Thus, in the presence of sucrose, glucose, sucralose and sorbitol, the biofilm mass was quantified up to 96 h, the pH of the spent culture media was measured, the expression of biofilm-related genes was determined, and demineralization challenge experiments were conduct in enamel fragments. The presence of sugars or sugar substitutes profoundly affected the expression of *spaP*, *gtfB*, *gtfC*, *gbpB*, *ftf*, *vicR* and *vicX* in either biofilm or planktonic cells. The substitution of sucrose induced a down-regulation of most genes involved in sucrose-dependent colonization in biofilm cells. When the ratio between the expression of biofilm and planktonic cells was considered, most of those genes were down-regulated in biofilm cells in the presence of sugars and up-regulated in the presence of sugar substitutes. However, sucralose but not sorbitol fulfilled the purpose of reducing the cariogenic potential of the diet since it induced the biofilm formation with the lowest biomass, did not change the pH of the medium and led to the lowest lesion depth in the cariogenic challenge.

Streptococcus mutans is considered the primary etiologic agent of dental caries. The ability of this pathogen to tolerate changes in the environmental pH and to form biofilms on the tooth surface allows *S. mutans* to survive and persist in the oral ecosystem.

S. mutans is a key contributor in the formation of biofilms associated with sucrose ingestion and dental caries, although other microorganisms may also be involved [Beighton, 2005; Kanasi et al., 2010]. *S. mutans* produces several surface molecules that are responsible for adhesion and colonization of teeth by sucrose-dependent and sucrose-independent mechanisms.

Protein SpaP (AgI/II or P1) is a protein anchored on the surface of *S. mutans* and one of the primary components associated with the adherence of *S. mutans* to saliva-coated hydroxyapatite, by interacting with several molecules (e.g. salivary agglutinin glycoprotein – SAG or GP340) [Russell and Mansson-Rahemtulla, 1989; Crow-

S.C. Durso and L.M. Vieira contributed equally to this study.

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Maria Regina L. Simionato
Department of Microbiology, Institute of Biomedical Sciences
University of São Paulo, Av. Prof. Lineu Prestes 1374
São Paulo SP 05508-000 (Brazil)
E-Mail mrsimion@usp.br

ley et al., 1999]. While this mechanism enables initial binding of this pathogen to the tooth surface, a selective advantage may be achieved by mechanisms dependent of sucrose that play an important role in biofilm accumulation and maturation by *S. mutans*.

In the presence of sucrose, extracellular enzymes such as glucosyltransferases (GtfB, GtfC and GtfD) are adsorbed to saliva-coated and/or bacterial surfaces and can generate large amounts of water-insoluble or -soluble glucans in situ [Venkitaraman et al., 1995; Vacca-Smith and Bowen, 1998; Bowen and Koo, 2011]. Glucan-binding proteins (GbpA, GbpB, GbpC and GbpD), non-enzymatic cell-associated proteins, can mediate the binding of *S. mutans* to glucans formed in situ, playing a key role in adhesion and biofilm formation of *S. mutans* in the presence of sucrose [Banas and Vickerman, 2003; Lemos et al., 2005; Bowen and Koo, 2011]. *S. mutans*, via fructosyltransferase (Ftf), also produces fructan from sucrose which functions primarily as extracellular nutritional reserve [Manly and Richardson, 1968; Shiroza and Kuramitsu, 1988; Burne et al., 1996]. Nevertheless, there is evidence that fructan also contributes to plaque formation, acting as binding sites for the accumulation of cariogenic bacteria [Rozen et al., 2001, 2004]. Although glucans or fructans are high-molecular-weight homopolymers of glucose or fructose, they are barely synthesized in the presence of glucose or fructose as the sole dietary carbohydrates, because *S. mutans* employs the energy released from the splitting of the glycosidic bond from sucrose to growing polysaccharide chains [Kiska and Macrina, 1994; Heyer et al., 1998; Monchois et al., 1999; Argimon et al., 2013].

After initial adhesion to the tooth surface, various genes are required for adaptation of *S. mutans* in developing biofilms. Bacterial cells in biofilms display phenotypic traits distinct from planktonic cells that are accompanied by significant changes in the gene expression profile [Costerton et al., 1987; Shemesh et al., 2007]. Among genes that encode regulatory proteins, *vicX* and *vicR* modulate adhesion, biofilm formation and tolerance to oxidative stress in *S. mutans* [Senadheera et al., 2007].

To avoid the role of sucrose on the virulence factors of *S. mutans*, the use of sugar substitutes is commonly suggested by dental clinicians because they lead to lower or no production of acids and are not substrates for glucan or fructan production, reducing the pathogenic potential of dental plaque. Among the most popular sugar substitutes are sucralose and sorbitol. Sucralose (1′,4′,6′-trideoxy-trichloro-galactosucrose) has qualities that enable it to be a very good choice for sucrose substitution. Sensory studies showed that the aftertaste of sucralose is similar to that of sucrose as far as sweetness is concerned [Horne et al., 2002; Kuhn et al., 2004]. Sucralose is a non-nutritive sweetener, 600 times sweeter than sucrose, and appears to be metabolically inert in many oral strains [Young and Bowen, 1990; Grice and Goldsmith, 2000]. It is poorly absorbed in the gastrointestinal tract, being considered safe for teeth and for human consumption [Grice and Goldsmith, 2000; Grotz and Munro, 2009].

Sorbitol is the sugar alcohol most often added to foods and its sweetness is about 60–70% that of sucrose. Sorbitol is caloric and accepted as being non-cariogenic because although it can be metabolized by few oral bacteria, including *S. mutans*, the production of acids is slow and it is not a substrate for the production of extracellular polysaccharides [Birkhed et al., 1984; Matsukubo and Takazoe, 2006].

Our objective was to investigate whether sugar substitutes considered safe for teeth have an effect on the virulence potential of *S. mutans* biofilms since sugar substitutes are widely consumed. In this study we investigate the influence of sugar substitutes – sucralose and sorbitol – in *S. mutans* biofilm formation and development by determining the biomass, the pH of spent culture media, the expression of biofilm-related genes and the lesion depth in enamel fragments. We were able to demonstrate that the sugar substitutes can influence (1) biofilm mass, (2) the expression of biofilm-related genes, and (3) the lesion depth in enamel fragments induced by biofilm of *S. mutans*. Our results suggest that between the tested sugar substitutes, sucralose, but not sorbitol, has the potential to undermine the virulence potential of *S. mutans* biofilm.

Materials and Methods

Bacteria and Culture Conditions

The *S. mutans* strain UA159 was selected for the study because it is a serotype c strain, the most common serotype found in the oral cavity, and the sequence of its genome is available [Ajdić et al., 2002]. *S. mutans* UA159 was grown overnight in Tryptic Soy Broth (TSB) (Difco Laboratories) at 37°C in a CO_2 Chamber (10% CO_2; Shel Lab).

Quantification of Biofilms Formed by S. mutans in the Presence of Sucrose, Glucose, Sucralose or Sorbitol

In order to determine the conditions for expression analysis, the biomass was evaluated from biofilms growing in the presence of each sugar or sugar substitute and the pH was recorded at the end of each time point. The biofilms were developed on the surface of 9 mm diameter glass coverslips (Knittel, Bielefeld, Germany) placed in 48-well microtiter plates (Biofill, Allentown, Pa., USA). *S. mutans* was grown overnight in TSB at 37°C in a CO_2 chamber. A subculture was established by diluting the culture in pre-warmed and pre-reduced

Table 1. Oligonucleotide primers used in RT-qPCR assays

Primers	Primer sequence (5′–3′)	Amplicon size (base pairs)	Reference
16SrRNAF 16SrRNAR	CTTACCAGGTCTTGACATCCCG ACCCAACATCTCACGACACGAG	111	Senadheera et al. [2007]
spaPF spaPR	GACTTTGGTAATGGTTATGCATCAA TTTGTATCAGCCGGATCAAGTG	101	Shemesh et al. [2007]
gtfBF gtfBR	ACACTTTCGGGTGGCTTG GCTTAGATGTCACTTCGGTTG	127	Senadheera et al. [2007]
gtfCF gtfCR	CCAAAATGGTATTATGGCTGTCG TGAGTCTCTATCAAAGTAACGCAG	136	Senadheera et al. [2007]
gbpBF gbpBR	AGCAACAGAAGCACAACCATCAG CCACCATTACCCCAGTAGTTTCC	150	Senadheera et al. [2007]
ftfF ftfR	ATTGGCGAACGGCGACTTACTC CCTGCGACTTCATTACGATTGGTC	103	Senadheera et al. [2005]
vicRF vicRR	CGCAGTGGCTGAGGAAAATG ACCTGTGTGTGTCGCTAAGTGATG	157	Senadheera et al. [2007]
vicXF vicXR	TGCTCAACCACAGTTTTACCG GGACTCAATCAGATAACCATCAGC	127	Senadheera et al. [2007]

TSB to an optical density of 0.3 at 600 nm (OD_{600}). The culture was incubated until it reached OD_{600} = 0.8 (1×10^{10} cfu/ml), distributed into four tubes and centrifuged for 5 min at 5,000 *g*. The cells were resuspended at the original volume in Tryptic Soy Broth Without Dextrose (TSBwoD) (Difco Laboratories; pH 6.9 ± 0.05) supplemented with sucrose, glucose, sucralose or sorbitol (0.4% w/v). From the standardized suspensions, aliquots of 500 µl were deposited in each well in quintuplicate. The biofilms were grown at the same culture conditions during 24, 48, 72 and 96 h [Wen et al., 2010], and the media was renewed every 24 h. For that, the planktonic fraction was collected and centrifuged for 5 min at 5,000 *g*, the pellet was resuspended in fresh medium and transferred to the respective wells, and the pH of the spent medium was recorded. After each time period, the biofilm was stained with 100 µl of 0.4% safranin for 15 min at room temperature. After washing three times by immersion in distilled water, the dye bound to biofilm cells was eluted in 200 µl of 95% ethanol for 15 min. Three aliquots of 50 µl of the de-staining solution from each well were transferred to a 96-well plate and the OD_{490} was measured on a plate reader (BioRad, Foster City, Calif., USA). The experiments were repeated three times. The differences in biofilm formation between samples were determined by Student's t test and considered significant when the p value was <0.05.

Growth of Biofilms for Gene Expression Analysis

Biofilms were grown on the surface of sterile glass slides in Coplin jars as previously described [Wen and Burne, 2002], in the same conditions established above in TSBwoD supplemented with sucrose, glucose, sucralose or sorbitol (0.4% w/v). The glass slides containing the biofilms were aseptically transferred daily to another Coplin jar containing fresh medium for a total of 4 days. Also, planktonic fraction was centrifuged, resuspended in fresh medium and inoculated in the jars to ensure the paired conditions between biofilm and planktonic cells.

RNA Isolation

After 4 days of growth, the biofilm (cells adhering to the glass slides) and the planktonic fraction (cells present in the spent culture media) were used for RNA isolation. For that, the planktonic fraction was removed, centrifuged and immediately resuspended in 1 ml TRIzol (Invitrogen) in screw-capped tubes of 2.0 ml (Sarstedt) containing 0.7 ml of glass beads (0.1 mm diameter; Sarstedt). The biofilm cells were also immediately removed from the slides with cell culture scrappers in the presence of 1.0 ml of TRIzol and transferred to the same type of tubes. The suspensions were transferred to a Mini BeadBeater homogenizer (Biospec Products) and homogenized for 6 cycles of 20 s at 4°C, with intervals of 1 min on ice. The suspensions were transferred to 50 ml Falcon tubes, added to 9.0 ml of TRIzol and mixed for 1 min. The following isolation of RNA was done as recommended by the manufacturer. To remove any DNA contamination, the RNA samples were treated with DNase I (Invitrogen) in solution and purified in RNeasy columns (Qiagen) with an additional DNase I treatment on-column. The samples were electrophoresed in agarose gel to verify the integrity of RNA and tested for amplification with primers for *S. mutans* 16S rRNA gene, in order to identify any contamination by genomic DNA. Three independent and paired samples of RNA were obtained from biofilm and planktonic cells to be tested for gene expression.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The cDNA was synthesized from 2 µg of total RNA, using Random Hexamer primers (3 mg/ml), and SuperScript III Reverse Transcriptase (200 U/µl) (Invitrogen) according to the instructions of the manufacturer. The reaction was carried out at 42°C for 16 h. RT-qPCR was performed in the iCycler iQ Real Time detection system (BioRad) using gene-specific primers (table 1). The

reactions (25 µl) were performed using 2 µl of cDNA samples as templates in a mixture containing 1× iQ SYBR Green Supermix (BioRad) and forward and reverse specific primers (200 nM). A standard thermal cycling program was used for amplification and detection of the fluorescent signal and the melting curve profile was analyzed to verify the primer specificity [Simionato et al., 2006].

The level of 16S rRNA transcripts in each sample was used to normalize the overall gene expression. There was no significant difference in the expression of 16S rRNA gene for all the conditions and samples tested. Each assay was performed with three independent RNA samples in duplicate (n = 6). The gene expression of biofilm and planktonic *S. mutans* cells in response to sucrose, glucose, sucralose and sorbitol was calculated using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001]. The fold change of each transcript was calculated and the results were expressed in relation to sucrose, the best substrate for biofilm formation by *S. mutans*. The ratio between the gene expression of cells in biofilm and the planktonic environment was also determined. Student's t test was used to calculate the significance of the difference between the mean expressions of the samples. Genes of *S. mutans* differentially expressed by the conditions with a p value <0.05 and at least 1.5-fold change were considered of biological and statistical significance as recommended by several studies [Peart et al., 2005; Simionato et al., 2006; Dalman et al., 2012; Van Peer et al., 2012].

In vitro Enamel Demineralization Induced by Sugars and Sugar Substitutes

To estimate the lesion depth on enamel fragments, optical coherence tomography (OCT) was used as previously described [Azevedo et al., 2011]. Human molar impacted teeth were removed, cleaned with pumice paste and water and stored in distilled water at 4°C until use. Approval was obtained from the Ethics Committee (University of São Paulo Dental School, FOUSP, protocol #11/2011). The teeth were previously examined with the help of a magnifying lens and all teeth showing enamel defects were excluded. Buccal surfaces were sectioned from human molars and the fragments were randomly distributed among four experimental groups. Half of the enamel area was covered with acid-resistant nail varnish and the fragments were assembled in structures that were placed in 50 ml centrifuge tubes sterilized with gamma ray irradiation (25 kGy). Briefly, for the demineralization challenge, biofilms of *S. mutans* were developed in 40 ml of TSBwoD supplemented by sucrose, glucose, sucralose or sorbitol (0.4% w/v) on the surface of fragments of enamel (about 3 × 3 mm). The cariogenic challenge was done for 7 days and the medium was renewed every day by transferring the structures to other tubes containing fresh medium. After the incubation period, the biofilm grown on the surface of the enamel was carefully removed and the specimens were washed with deionized water. Immediately before the OCT reading, the nail varnish was mechanically removed from the surfaces of the fragments.

Optical Coherence Tomography

In the OCT system (SR930, Thorlabs Inc., Newton, Mass., USA), a super-luminescent light-emitting diode (λ = 930 nm) provided A and B scans with 4.0- and 6.0-µm resolution, respectively. The system was able to produce up to 4 frames per second, thus providing real-time images (2,000 × 512 pixels; equivalent to a width × height area of 6,000 × 1,581 µm^2). The images were acquired from the central regions of the exposed windows, generating images from both exposed and varnish-protected (control) surfaces. The images of the caries-affected enamel were obtained by OCT for exposure of biofilm to sucrose, glucose, sucralose or sorbitol and were compared according to demineralization depth. If any tissue loss occurred due to the demineralization process, the amount of lost tissue was included in the calculation, with the adjacent sound enamel as the initial reference.

Statistical Analysis

Statistical analyses were performed using the Microsoft Excel software. Student's t test was used for each individual comparison between glucose or sugar substitutes outcomes against sucrose. For all analyses a probability of $p < 0.05$ was considered significant.

Table 2. Quantification of biofilms formed by *S. mutans* in the presence of sucrose, glucose, sucralose or sorbitol (OD_{490}) at the end of each time point

Time	Sucrose	Glucose	Sucralose	Sorbitol
24 h	0.232±0.03	0.142±0.04[a]	0.153±0.04[a]	0.186±0.09[a]
48 h	0.290±0.06*	0.174±0.04*, [a]	0.166±0.05[a]	0.212±0.03*, [a]
72 h	0.558±0.05*	0.225±0.07*, [a]	0.198±0.05*, [a]	0.325±0.07*, [a, b, c]
96 h	0.603±0.04	0.253±0.06[a]	0.234±0.06*, [a]	0.381±0.04[a, b, c]

Figures are mean ± SD. Values followed by an asterisk or a letter represent a significant difference by Student's t test ($p < 0.05$). * Significant difference within the same sugar/sugar substitute between consecutive time points. [a] Significant difference of sugar/sugar substitute to sucrose at the same time point. [b] Significant difference of sugar/sugar substitute to glucose at the same time point. [c] Significant difference of sugar/sugar substitute to sucralose at the same time point.

Results

The evaluation of the biomass in 48-well plates showed that in 72 h the biofilm reached its maximum biomass in the presence of sucrose, glucose and sorbitol. In the presence of sucralose the maximum biomass was reached in 96 h. Sucrose was the supplement that allowed a higher biomass than glucose and sugar substitutes at each time point. After 72 and 96 h, sorbitol showed higher formation than glucose and sucralose, which were not statistically different from each other (table 2).

The pH recorded in the spent culture media plunged after 24 h of growth in the presence of sucrose, glucose and sorbitol (from 6.9 to about 4.8). In the presence of sucrose, the lowest pH recorded was reached in 72 h of growth (4.1 ± 0.06), and in the presence of glucose and sorbitol the lowest pH recorded was reached in 48 h (4.4 ±

Table 3. pH recorded in the planktonic fraction at the end of each time point of *S. mutans* biofilms developed in the presence of sucrose, glucose, sucralose or sorbitol

Time	Sucrose	Glucose	Sucralose	Sorbitol
24 h	4.8±0.05	4.7±0.05	6.8±0.05[a, b]	4.7±0.05[c]
48 h	4.4±0.10*	4.4±0.05*	6.9±0.04[a, b]	4.3±0.08*, [c]
72 h	4.1±0.06*	4.2±0.04	6.9±0.05[a, b]	4.3±0.05[c]
96 h	4.0±0.04	4.0±0.05	6.7±0.05[a, b]	4.3±0.04[c]

Figures are mean ± SD. Values followed by an asterisk or a letter represent a significant difference by Student's t test ($p < 0.05$). * Significant difference within the same sugar/sugar substitute between consecutive time points. [a] Significant difference of sugar/sugar substitute to sucrose at the same time point. [b] Significant difference of sugar/sugar substitute to glucose at the same time point. [c] Significant difference of sugar/sugar substitute to sucralose at the same time point.

Table 4. RT-qPCR analysis of selected genes of *S. mutans* expressed in biofilm or planktonic cells growing in the presence of glucose, sucralose or sorbitol

	Biofilm			Planktonic		
	glucose	sucralose	sorbitol	glucose	sucralose	sorbitol
spaP	2.3	2.0	5.2	3.0	3.2	14.1
gtfB	–1.6	ND	–2.1	ND	3.0	2.8
gtfC	–2.7	–4.0	ND	–2.6	–2.0	6.8
gbpB	–3.3	–1.8	ND	–3.6	1.7	3.5
ftf	–7.3	ND	–5.7	–12.4	ND	–4.6
vicR	ND	ND	1.9	ND	3.1	3.3
vicX	ND	ND	2.5	–1.5	2.3	3.7

The transcription profile of *S. mutans* cells in the presence of sucrose was used as control, and fold changes of each gene for each condition were calculated in relation to the gene expression in the presence of sucrose.

ND = Not different (p value > 0.05 or fold change < 1.5).

0.05 and 4.3 ± 0.08, respectively) and remained with similar pH values up to 96 h. However, the pH of the spent media did not change over time when it was supplemented with sucralose (about 6.8) (table 3).

The presence of sucrose, glucose, sucralose or sorbitol profoundly affected the expression of biofilm-related genes of *S. mutans*. The fold change in the gene expression of biofilm and planktonic cells in relation to sucrose is listed in table 4. The gene *spaP* is the only one in biofilm and planktonic cells that is differentially expressed in the presence of glucose, sucralose or sorbitol in relation to sucrose. In the presence of glucose or sucralose the level of the transcripts of genes *vicR* and *vicX* in biofilm cells was similar to sucrose, while in the presence of sorbitol both genes were up-regulated. Genes involved in colonization based on extracellular polysaccharide such as *gtfB*, *gtfC*, *gbpB*, and *ftf*, when differentially expressed in relation to sucrose, were down-regulated in biofilm cells in the absence of sucrose. The fold change between biofilm and planktonic cells was evaluated for each condition as well, and when differentially expressed, genes were down-regulated in biofilm cells in the presence of sugars and up-regulated in the presence of sugar substitutes (table 5). The expression of biofilm and planktonic cells in response to sucralose was similar for most of the genes.

Table 5. Influence of the biofilm or the planktonic environment in the expression of the selected genes of *S. mutans* cells in the presence of sucrose, glucose, sucralose or sorbitol

Gene	Sucrose B/P	Glucose B/P	Sucralose B/P	Sorbitol B/P
spaP	–1.5	ND	ND	1.8
gtfB	–2.3	–1.5	1.5	2.5
gtfC	–2.3	–2.2	ND	2.3
gbpB	–2.5	–2.8	ND	ND
ftf	ND	–2.5	ND	ND
vicR	–2.0	ND	ND	ND
vicX	ND	–1.8	ND	ND

ND = Not different (p value > 0.05 or fold change < 1.5); B/P = ratio of gene expression between biofilm and planktonic cells.

The lesion depth on enamel fragments was different after exposure to glucose, sucralose and sorbitol in relation to sucrose, as presented in figure 1. As expected, sucrose is the substrate that induced the highest mineral loss, while sucralose led to the lowest demineralization depth. Differences in lesion depth were observed for glucose and sucralose as well as between sucralose and sorbitol, but surprisingly in the presence of glucose and sorbitol there was no difference in response to the cariogenic challenge.

Discussion

The metabolic activities of *S. mutans* in dental plaque are critical factors for the pathogenesis of dental caries. Although other microorganisms in oral biofilms may be considered cariogenic [Garcia-Godoy and Hicks, 2008], *S. mutans* has several advantageous properties, including rapid transportation and fermentation of dietary carbo-

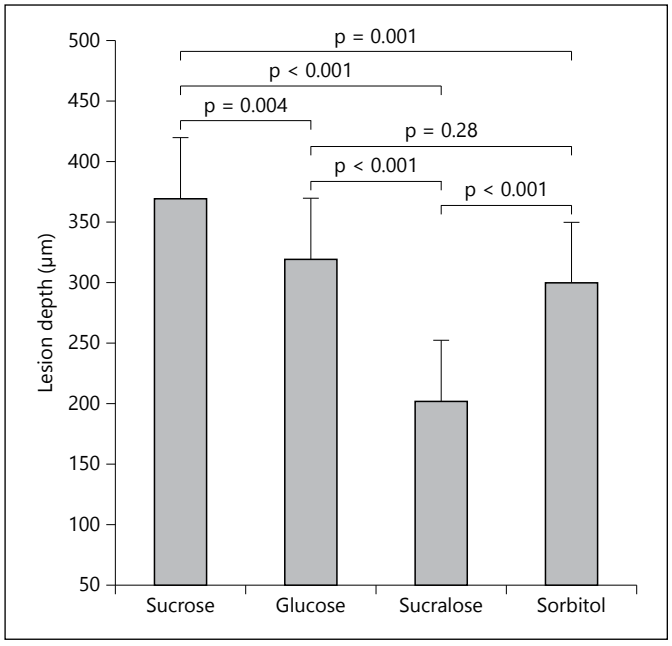

Fig. 1. Lesion depth (µm) estimated by OCT after 7-day exposure of enamel fragments to *S. mutans* growing in the presence of sucrose, glucose, sucralose or sorbitol. The results are mean values (n = 4) and the error bars represent standard deviations (Student's t test; significant difference when $p < 0.05$).

hydrates producing lactic, formic, acetic and propionic acids, extra- and intracellular polysaccharide synthesis, and carbohydrate metabolism under stress conditions [Loesche, 1986; Lemos et al., 2005]. Substitutes of sucrose are frequently employed by the food and oral hygiene industries because several virulent properties of cariogenic bacteria are observed mostly in the presence of sucrose.

The addition of sucrose to the medium leads to the highest biofilm mass, followed by sorbitol, glucose and sucralose. A change in the recorded pH of spent culture media could also be observed. Biofilms growing exposed to sucralose were not able to change their initial pH, which was recorded below 4.8 in spent culture media of biofilms growing exposed to sucrose, glucose or sorbitol. The variation of the recorded pH in the spent culture media is an outcome of the bacterial metabolism under a particular condition, and it is important to evaluate it in the experiments since the intake of sugars or sugar substitutes may lead to temporary changes in the environment of dental plaque and will certainly modify the gene expression profile of biofilm and planktonic cells.

The fold change in gene expression of *S. mutans* growing in the presence of glucose, sucralose and sorbitol in relation to sucrose was calculated. During biofilm formation, biofilm cells demonstrate a different phenotype from planktonic partners, showing that changes in environmental conditions can significantly alter the pattern of gene expression of microorganisms [Wen and Burne, 2002; Marsh and Devine, 2011]. Most of the studies focused their attention only on the expression of biofilm cells. The gene expression of planktonic cells is also important to be investigated because it can reflect the potential of free cells to colonize new surfaces, either developing a new biofilm or attaching to an existing biofilm. Thus, the ratio of the expression of a particular gene in biofilm and planktonic cells was also determined.

The genes *vicR* and *vicX* encode regulatory proteins that can modulate virulence attributes in *S. mutans*, such as *gtfB/C* expression, biofilm formation and oxidative stress tolerance [Senadheera et al., 2005, 2007]. RT-qPCR data showed that when exposed to sorbitol, both genes were more expressed in biofilm cells in relation to sucrose, glucose and sucralose. This phenomenon could explain the larger biofilm mass observed for biofilms exposed to sorbitol than those exposed to glucose and sucralose, although a larger biomass did not necessarily induce more enamel demineralization.

Another biofilm-related gene, *spaP*, encodes a surface protein that binds to the salivary agglutinin present in the acquired pellicle that coats the teeth surfaces [Russell and Mansson-Rahemtulla, 1989] and enables *S. mutans* to adhere to tooth surfaces in the absence of sucrose [Jenkinson and Demuth, 1997; Jenkinson and Lamont, 1997]. The transcription of *spaP* in *S. mutans* is dependent of the condition that the bacteria are growing [Wen et al., 2010]. Under a diet rich in sucrose, a mutant strain had a cariogenic behavior similar to the parental strain, probably because the colonization using mechanisms dependent on glucan production and binding supplanted the loss of gene *spaP* in the mutant strain [Bowen et al., 1991]. On the other hand, under a low sucrose diet in an in vivo model, a *spaP* mutant caused a lower number of carious lesions than the parental strain, suggesting a role of this protein in the virulence of *S. mutans* [Crowley et al., 1999]. Our data indicate that sucrose-independent mechanisms in biofilm formation of *S. mutans* may be relevant in biofilm growth of *S. mutans* in the presence of sugar substitutes, notably in the presence of sorbitol. Although the cariogenic potential of sorbitol has been considered low [Bowen et al., 1990; Bowen and Pearson, 1992], clinical studies reported an increase in *S. mutans* counts in dental plaque following consumption or mouth rinses with sorbitol [Soderling et al., 1989; Kalfas et al., 1990; Giertsen et al., 2011].

Other genes involved in adhesion/colonization of oral sites using extracellular polysaccharide – *gbpB*, *gtfB*, *gtfC* and *ftf* – when differentially expressed were less expressed in biofilm cells in the absence of sucrose, while in the planktonic fraction the expression of these genes was dependent on the sugar or sugar substitute present in the growth medium. Glucosyltransferases and glucan-binding proteins of *S. mutans* are known to be differentially expressed in response to environmental conditions, such as growth medium, carbohydrate source and concentration, and pH [Li and Burne, 2001; Banas and Vickerman, 2003; Shemesh et al., 2007; Klein et al., 2010]. Our data indicate that genes involved in sucrose-dependent biofilm formation are regulated by sugar substitutes as well, which may contribute for the measured biofilm growth.

A different outline in the biofilm/planktonic expression ratio of *gbpB*, *gtfB*, *gtfC* and *ftf* has been observed when *S. mutans* is growing in the presence of sucrose or glucose versus when growing in the presence of sucralose or sorbitol. Young and Bowen [1990] reported that the activity of Gtf and Ftf in solution is reversibly inhibited by the presence of sucralose. The same outcome was demonstrated by sucralose, but not by sorbitol or xylitol, in Gtfs adsorbed to a saliva-coated hydroxyapatite [Wunder and Bowen, 1999]. Our data demonstrate that *gbpB*, *gtfB*, *gtfC* and *ftf*, which are involved in adhesion/colonization using extracellular polysaccharide, are more expressed in planktonic cells than in biofilm cells exposed to sucrose or glucose, which may indicate that those genes are important for the planktonic cells to be set to attach to other surfaces in this experimental condition, probably the biofilm cells. Although is very well known that Ftf converts sucrose into a homopolymer of fructose [Birkhed et al., 1979; Shiroza and Kuramitsu, 1988; Zeng and Burne, 2013], further studies are still necessary for a better understanding of the role of *ftf* when *S. mutans* is exposed to sugar substitutes in the absence of sucrose.

It is very well known that in the presence of sucrose, *S. mutans* produces large amounts of glucans and fructan that combined with Gbps constitute important factors in biofilm formation. Additionally, *S. mutans* produces varied acids growing in the presence of sugars. It is well known that the recipe biofilm plus acid production is a key contributor to caries formation. The demineralization challenge showed that sucrose promoted the highest mineral loss. Interestingly, glucose and sorbitol promoted a similar mineral loss. A higher biomass was observed for *S. mutans* exposed to sorbitol than exposed to glucose. In addition, both substrates induced low pH readings after 24 h of growth, similar to pH readings observed for sucrose-exposed biofilm. Also, though no significant difference ($p = 0.403$) was observed between the biofilm masses of *S. mutans* in the presence of glucose or sucralose, the mineral loss was higher in the presence of glucose than in the presence of sucralose ($p < 0.001$). Thus, our data may indicate that the low pH recorded in the spent culture media of biofilms exposed to glucose and sorbitol was one of the determinant factors for the similar lesion depth observed in the enamel fragments for both carbohydrates.

While sorbitol is not a substrate for the formation of glucan or fructan by *S. mutans* [Argimon et al., 2013], the molecular mechanisms involved in biofilm formation in the presence of this sugar alcohol are not completely known. This study showed that sorbitol supported biofilm formation with lower biomass than sucrose biofilm but higher than glucose and sucralose biofilms. Moreover, the final pH value was similar to the pH readings of biofilms growing in the presence of sucrose and glucose, which is a key factor for dental demineralization. These properties do not fulfill the desired requirements in completely reducing the cariogenic potential of dental plaque, as expected for a recommended sugar substitute.

Surprisingly, a slight demineralization was observed in the enamel fragments exposed to *S. mutans* growing in the presence of sucralose. The pH of the spent culture media did not change throughout time (96 h) although the recorded pH was done in a different system (48-well plate). Nevertheless, the recorded pH determined in our study is similar to what has been reported in several previous studies. However, in this in vitro study we did not use buffered media and bacteria were not refrained by the competition of other species. In a recent report it has been shown that the biofilm architecture is heterogeneous, and despite buffered media, a range of acidic regions were found in the interface of the microcolony with the hydroxyapatite substrate, which may have implications for cariogenesis studies [Xiao et al., 2012]. Thus, we believe that further studies of the architecture, structure and the matrix of sucralose biofilms are necessary to better understand the reason of this small grade of demineralization found in the presence of sucralose.

The substitution of sucrose for sugar substitutes with the purpose of reducing the cariogenic potential of the diet should be taken very carefully. The results of this study showed that sucralose may have the advantage of inducing less biofilm formation, not contributing to the decrease of pH, and thereby may not allow the selection of *S. mutans* in biofilm. Also, the profile of gene expression involved in the development of virulent dental

plaque in *S. mutans* showed that the presence of sucralose, but not sorbitol, probably interferes in the metabolism of *S. mutans*, leading to a less harmful dental plaque.

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Author Contributions

S.C. Durso and L.M. Vieira contributed equally to this paper, growing biofilms under different conditions and conducting the experiments of gene expression. J.N.S. Cruz performed the biomass quantification and the cariogenic challenge. C.S. Azevedo prepared the enamel fragments for cariogenic challenge. P.H. Rodrigues conceived and designed the experiments, analyzed the data and wrote the paper. M.R.L. Simionato conceived and designed the experiments, analyzed the data and wrote the paper.

Disclosure Statement

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

- Ajdić D, McShan WM, McLaughlin RE, Savić G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H, Lin S, Qian Y, Li S, Zhu H, Najar F, Lai H, White J, Roe BA, Ferretti JJ: Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc Natl Acad Sci USA 2002;99:14434–14439.
- Argimon S, Alekseyenko AV, DeSalle R, Caufield PW: Phylogenetic analysis of glucosyltransferases and implications for the coevolution of mutans streptococci with their mammalian hosts. PLoS One 2013;8:e56305.
- Azevedo CS, Trung LC, Simionato MR, Freitas AZ, Matos AB: Evaluation of caries-affected dentin with optical coherence tomography. Braz Oral Res 2011;25:407–413.
- Banas JA, Vickerman MM: Glucan-binding proteins of the oral streptococci. Crit Rev Oral Biol Med 2003;14:89–99.
- Beighton D: The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol 2005;33:248–255.
- Birkhed D, Edwardsson S, Kalfas S, Svensater G: Cariogenicity of sorbitol. Swed Dent J 1984;8:147–154.
- Birkhed D, Rosell KG, Granath K: Structure of extracellular water-soluble polysaccharides synthesized from sucrose by oral strains of *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguis* and *Actinomyces viscosus*. Arch Oral Biol 1979;24:53–61.
- Bowen WH, Koo H: Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. Caries Res 2011;45:69–86.
- Bowen WH, Pearson SK: The effects of sucralose, xylitol, and sorbitol on remineralization of caries lesions in rats. J Dent Res 1992;71:1166–1168.
- Bowen WH, Pearson SK, Falany JL: Influence of sweetening agents in solution on dental caries in desalivated rats. Arch Oral Biol 1990;35:839–844.
- Bowen WH, Schilling K, Giertsen E, Pearson S, Lee SF, Bleiweis A, Beeman D: Role of a cell surface-associated protein in adherence and dental caries. Infect Immun 1991;59:4606–4609.
- Burne RA, Chen YY, Wexler DL, Kuramitsu H, Bowen WH: Cariogenicity of *Streptococcus mutans* strains with defects in fructan metabolism assessed in a program-fed specific-pathogen-free rat model. J Dent Res 1996;75:1572–1577.
- Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ: Bacterial biofilms in nature and disease. Annu Rev Microbiol 1987;41:435–464.
- Crowley PJ, Brady LJ, Michalek SM, Bleiweis AS: Virulence of a *spaP* mutant of *Streptococcus mutans* in a gnotobiotic rat model. Infect Immun 1999;67:1201–1206.
- Dalman MR, Deeter A, Nimishakavi G, Duan ZH: Fold change and p-value cutoffs significantly alter microarray interpretations. BMC Bioinformatics 2012;13(suppl 2):S11.
- Garcia-Godoy F, Hicks MJ: Maintaining the integrity of the enamel surface: the role of dental biofilm, saliva and preventive agents in enamel demineralization and remineralization. J Am Dent Assoc 2008;139(suppl):25S–34S.
- Giertsen E, Arthur RA, Guggenheim B: Effects of xylitol on survival of mutans streptococci in mixed-six-species in vitro biofilms modelling supragingival plaque. Caries Res 2011;45:31–39.
- Grice HC, Goldsmith LA: Sucralose – an overview of the toxicity data. Food Chem Toxicol 2000;38(suppl 2):S1–S6.
- Grotz VL, Munro IC: An overview of the safety of sucralose. Regul Toxicol Pharmacol 2009;55:1–5.
- Heyer AG, Schroeer B, Radosta S, Wolff D, Czapla S, Springer J: Structure of the enzymatically synthesized fructan inulin. Carbohydr Res 1998;313:165–174.
- Horne J, Lawless HT, Speirs W, Sposato D: Bitter taste of saccharin and acesulfame-K. Chem Senses 2002;27:31–38.
- Jenkinson HF, Demuth DR: Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. Mol Microbiol 1997;23:183–190.
- Jenkinson HF, Lamont RJ: Streptococcal adhesion and colonization. Crit Rev Oral Biol Med 1997;8:175–200.
- Kalfas S, Maki Y, Birkhed D, Edwardsson S: Effect of pH on acid production from sorbitol in washed cell suspensions of oral bacteria. Caries Res 1990;24:107–112.
- Kanasi E, Johansson I, Lu SC, Kressin NR, Nunn ME, Kent R Jr, Tanner AC: Microbial risk markers for childhood caries in pediatricians' offices. J Dent Res 2010;89:378–383.
- Kiska DL, Macrina FL: Genetic analysis of fructan-hyperproducing strains of *Streptococcus mutans*. Infect Immun 1994;62:2679–2686.
- Klein MI, DeBaz L, Agidi S, Lee H, Xie G, Lin AH, Hamaker BR, Lemos JA, Koo H: Dynamics of *Streptococcus mutans* transcriptome in response to starch and sucrose during biofilm development. PLoS One 2010;5:e13478.
- Kuhn C, Bufe B, Winnig M, Hofmann T, Frank O, Behrens M, Lewtschenko T, Slack JP, Ward CD, Meyerhof W: Bitter taste receptors for saccharin and acesulfame K. J Neurosci 2004;24:10260–10265.
- Lemos JA, Abranches J, Burne RA: Responses of cariogenic streptococci to environmental stresses. Curr Issues Mol Biol 2005;7:95–107.
- Li Y, Burne RA: Regulation of the *gtfBC* and *ftf* genes of *Streptococcus mutans* in biofilms in response to pH and carbohydrate. Microbiology 2001;147:2841–2848.

Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001;25:402–408.

Loesche WJ: Role of *Streptococcus mutans* in human dental decay. Microbiol Rev 1986;50:353–380.

Manly RS, Richardson DT: Metabolism of levan by oral samples. J Dent Res 1968;47:1080–1086.

Marsh PD, Devine DA: How is the development of dental biofilms influenced by the host? J Clin Periodontol 2011;38(suppl 11):28–35.

Matsukubo T, Takazoe I: Sucrose substitutes and their role in caries prevention. Int Dent J 2006;56:119–130.

Monchois V, Willemot RM, Monsan P: Glucansucrases: mechanism of action and structure-function relationships. FEMS Microbiol Rev 1999;23:131–151.

Peart MJ, Smyth GK, van Laar RK, Bowtell DD, Richon VM, Marks PA, Holloway AJ, Johnstone RW: Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors. Proc Natl Acad Sci USA 2005;102:3697–3702.

Rozen R, Bachrach G, Bronshteyn M, Gedalia I, Steinberg D: The role of fructans on dental biofilm formation by *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus gordonii* and *Actinomyces viscosus*. FEMS Microbiol Lett 2001;195:205–210.

Rozen R, Bachrach G, Steinberg D: Effect of carbohydrates on fructosyltransferase expression and distribution in *Streptococcus mutans* GS-5 biofilms. Carbohydr Res 2004;339:2883–2888.

Russell MW, Mansson-Rahemtulla B: Interaction between surface protein antigens of *Streptococcus mutans* and human salivary components. Oral Microbiol Immunol 1989;4:106–111.

Senadheera MD, Guggenheim B, Spatafora GA, Huang YC, Choi J, Hung DC, Treglown JS, Goodman SD, Ellen RP, Cvitkovitch DG: A VicRK signal transduction system in *Streptococcus mutans* affects *gtfBCD*, *gbpB*, and *ftf* expression, biofilm formation, and genetic competence development. J Bacteriol 2005;187:4064–4076.

Senadheera MD, Lee AW, Hung DC, Spatafora GA, Goodman SD, Cvitkovitch DG: The *Streptococcus mutans vicX* gene product modulates *gtfB/C* expression, biofilm formation, genetic competence, and oxidative stress tolerance. J Bacteriol 2007;189:1451–1458.

Shemesh M, Tam A, Steinberg D: Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. Microbiology 2007;153:1307–1317.

Shiroza T, Kuramitsu HK: Sequence analysis of the *Streptococcus mutans* fructosyltransferase gene and flanking regions. J Bacteriol 1988;170:810–816.

Simionato MR, Tucker CM, Kuboniwa M, Lamont G, Demuth DR, Tribble GD, Lamont RJ: *Porphyromonas gingivalis* genes involved in community development with *Streptococcus gordonii*. Infect Immun 2006;74:6419–6428.

Soderling E, Makinen KK, Chen CY, Pape HR Jr, Loesche W, Makinen PL: Effect of sorbitol, xylitol, and xylitol/sorbitol chewing gums on dental plaque. Caries Res 1989;23:378–384.

Vacca-Smith AM, Bowen WH: Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. Arch Oral Biol 1998;43:103–110.

Van Peer G, Mestdagh P, Vandesompele J: Accurate RT-qPCR gene expression analysis on cell culture lysates. Sci Rep 2012;2:222.

Venkitaraman AR, Vacca-Smith AM, Kopec LK, Bowen WH: Characterization of glucosyltransferaseB, GtfC, and GtfD in solution and on the surface of hydroxyapatite. J Dent Res 1995;74:1695–1701.

Wen ZT, Burne RA: Functional genomics approach to identifying genes required for biofilm development by *Streptococcus mutans*. Appl Environ Microbiol 2002;68:1196–1203.

Wen ZT, Yates D, Ahn SJ, Burne RA: Biofilm formation and virulence expression by *Streptococcus mutans* are altered when grown in dual-species model. BMC Microbiol 2010;10:111.

Wunder D, Bowen WH: Action of agents on glucosyltransferases from *Streptococcus mutans* in solution and adsorbed to experimental pellicle. Arch Oral Biol 1999;44:203–214.

Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates JR 3rd, Heydorn A, Koo H: The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog 2012;8:e1002623.

Young DA, Bowen WH: The influence of sucralose on bacterial metabolism. J Dent Res 1990;69:1480–1484.

Zeng L, Burne RA: Comprehensive mutational analysis of sucrose-metabolizing pathways in *Streptococcus mutans* reveals novel roles for the sucrose phosphotransferase system permease. J Bacteriol 2013;195:833–843.